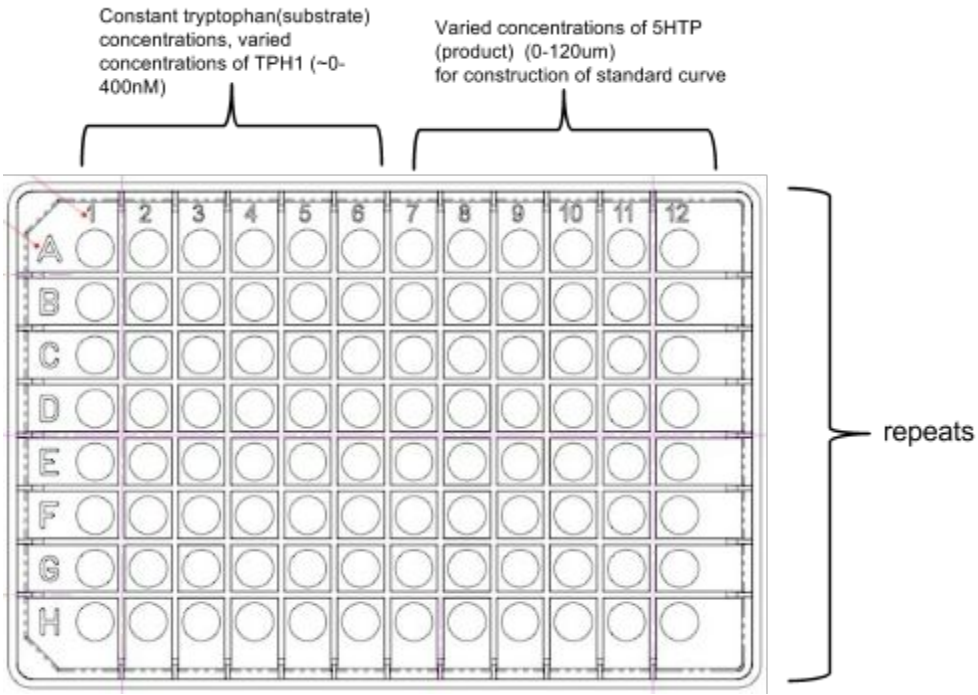
Assay for activity of tryptophan hydroxylase 1

General principle

Based on “A continuous fluorescence assay for Tryptophan Hydroxylase” Moran and Fitzpatrick, Analytical Biochemistry 266, 148–152 (1999)

A continuous fluorometric assay for tryptophan hydroxylase activity based on the different spectral characteristics of tryptophan and 5-hydroxytryptophan is presented. Hydroxylation of tryptophan at the 5-position results in a large increase in the fluorescence of the molecule. The assay selectively monitors the fluorescence yield of 5-hydroxytryptophan by exciting the reaction mix at 300 nm. The rate of increase of the emission signal was found to be directly proportional to the enzyme concentration. Inner filter effects due to quinonoid dihydropterin accumulation were eliminated by the inclusion of a thiol reductant. Activity measured using this assay method was found to be the same as that determined by established discontinuous HPLC assay methods.

The standard reaction conditions are: 60 uM tryptophan, 300 uM 6-methyltetrahydropterin, 200 mM ammonium sulfate, 7 mM DTT, 25 ug/ml catalase, 25 uM ferrous ammonium sulfate, 50 mM Mes, pH 7.0, with atmospheric oxygen, 0-400 nM (=0-102 ug) of TPH1



Reagents:

a. tryptophan
b. 6-methyltetrahydrobiopterin
c. ammonium sulphate
d. DTT
e. catalase
f. ferrous ammonium sulphate
g. MES hydrate
h. Tris
i. HCl
j. EDTA
k. 5-hydroxytryptophan

Assay measurements:

On the day of the assay:

1) Prepare 500ml stock of 100 mM MES buffer, pH7

- Dissolve 9.76 g of MES free acid (Mr = 195.2) in approx. 450 ml of pure water.
- Titrate to pH 7 with NaOH
- Make up volume to 500 ml with pure water

2) Prepare solutions of TPH1 and 5-hydroxytryptophan at varied concentrations (keep these on ice!):

	1	2	3	4	5
TPH1:	?	?	?	?	?
Mes buffer:	?	?	?	?	?
Total:	1 ml	1 ml	1 ml	1 ml	1 ml

Ideally we need 6.375 ug of TPH1 in solution 1, 12.75 ug in solution 3…, 102 ug in solution 5. These volumes should be decided on the evening before (after carrying out Bradford assay). //these will be filled up later//

	1	2	3	4	5
5-HTP	0.0083 mg (37.5 uM)	0.017 mg (75 uM)	0.033 mg (150 uM)	0.066 mg (300uM)	0.132 mg (600uM)
H2O:	1 ml	1 ml	1 ml	1 ml	1 ml

3) Prepare Solution A (Total volume = 40 ml) :

Required concentration*	**Source**	**Amount to be added**
100 mM Mes, pH 7.0	stock solution	40 ml
400 mM ammonium sulfate	powder	2.64 g
2 mM DTT	powder	15.4 mg
50 ug/ml catalase	powder	2.5 mg
50 uM ferrous ammonium sulfate	powder	0.98 mg

*These are the final concentrations after addition of TPH1 solutions to solution A

4) Prepare 5 falcon tubes, name them A+TPH1-1/2/3/4/5 and add 4 ml of solution A. Then add TPH1 solutions 1-5 to each of the tube. Add 5 ml of Mes buffer to the remaining 20 ml solution A and name it A+TPH1-0:

A+TPH1-0	**A+TPH1-1**	**A+TPH-2**	**A+TPH1-3**	**A+TPH1-4**	**A+TPH1-5**
20 ml Solution A + 5 ml Mes buffer	4 ml solution A + 1 ml TPH1-1	4 ml solution A + 1 ml TPH1-2	4 ml solution A + 1 ml TPH1-3	4 ml solution A + 1 ml TPH1-4	4 ml solution A + 1 ml TPH1-5

(keep in the fridge until used)

5) Prepare Solution B (Total volume = 50 ml) and solution C (Total volume = 50 ml) :

Solution B:

Required concentration	**Source**	**Amount to be added**
120 um tryptophan	powder	1.2 mg
600 um 6-MePH4	powder	7.62 mg
10 mM HCl	1 M solution	0.5 ml
12 mM DTT	powder	92.4 mg
ddH2O	-	49.5 ml

Solution C:

Required concentration	**Source**	**Amount to be added**
600 um 6-MePH4	powder	7.62 mg
10 mM HCl	1 M solution	0.5 ml
12 mM DTT	powder	92.4 mg

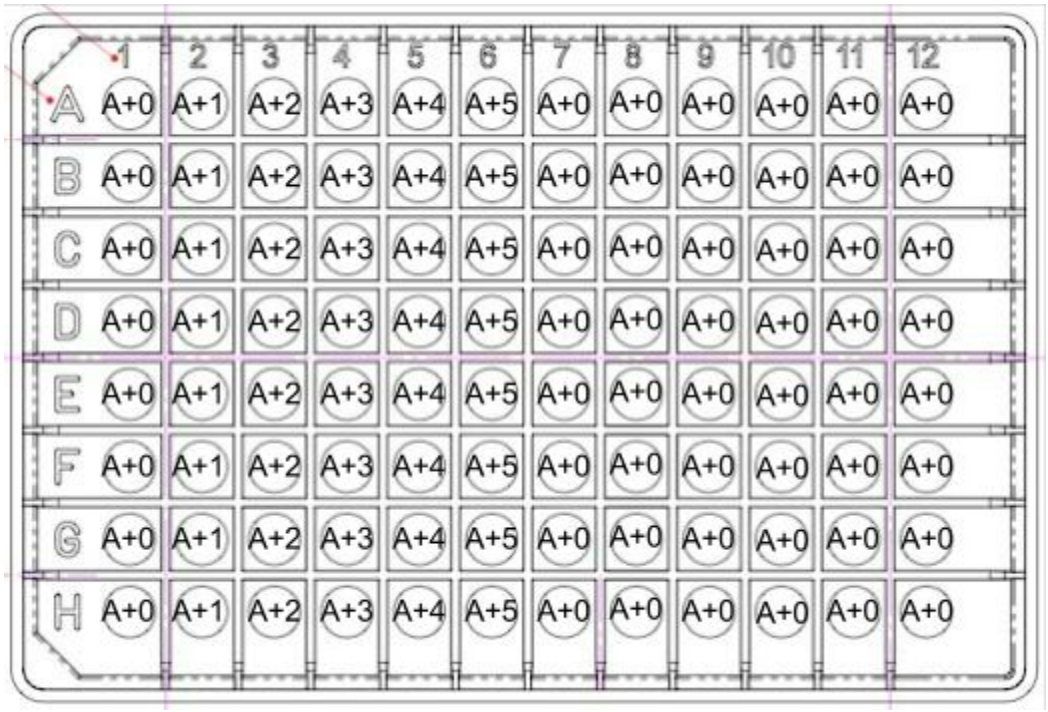
ddH2O	-	40 ml

6) Prepare 5 falcon tubes, name them C+5HTP-1/2/3/4/5 and add 4 ml of solution C. Then add 5HTP solutions 1-5 to each of the tube. Add 5 ml of ddH2O to the remaining 20 ml solution A and name it C+5HTP-0:

C+5HTP-0	**C+5HTP-1 (7.5 uM)**	**C+5HTP-2 (15 uM)**	**C+5HTP-3 (30uM)**	**C+5HTP-4 (60 uM)**	**C+5HTP-5 (120 uM)**
20 ml Solution C + 5 ml ddH2O	4 ml solution C + 1 ml 5HTP-1	4 ml solution C + 1 ml 5HTP-2	4 ml solution C + 1 ml 5HTP-3	4 ml solution C + 1 ml 5HTP-4	4 ml solution C + 1 ml 5HTP-5

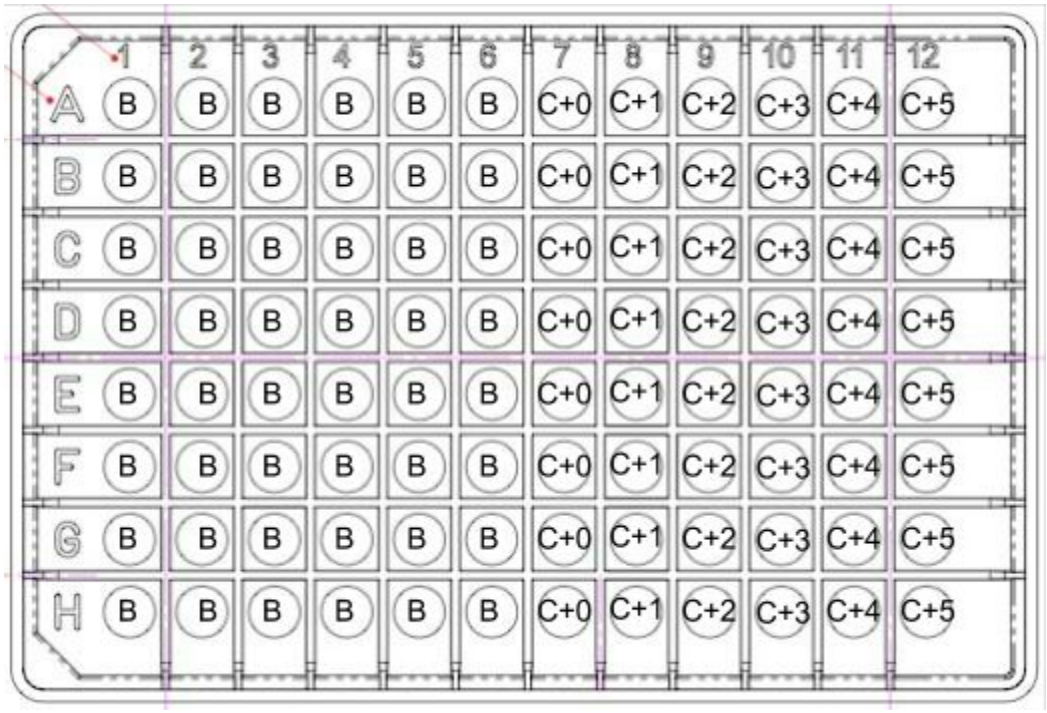
(keep in the fridge until used)

7) Pipette 150 ul of following solutions into 96 well plate:



8) Equilibrate for 2 mins at 15 C

9) Initiate assay by adding 150 ul of following solutions:



10. Measure activity on microplate reader by excitation at 300 nm and emission at 330 nm every 10 mins for 2 hours at 15 C (required for TPH1 stability)

11. Plot absorbance data for standard curve and for the TPH1 activity